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AWARD NUMBER: W81XWH-06-1-0702

TITLE: Characterization of Human Mammary Epithelial Stem Cells

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REPORT DATE: October 2009

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1 October 2009	Annual Summary	15 Sep 2006 – 14 Sep 2009

4. TITLE AND SUBTITLE

Characterization of Human Mammary Epithelial Stem Cells

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0702

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

British Columbia Cancer Agency
Vancouver, BC V5Z 1L3 Canada

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The mammary epithelium of normal adult female mice contains stem cells with extensive in vivo regenerative and self-renewal potential. Indirect evidence has suggested that analogous cells exist in the mammary glands of adult women, and are candidate targets for the first transforming mutations that lead to the evolution of breast cancer stem cells. The objective of this grant was to determine whether these hypothesized normal human mammary stem cells might be detected and quantified by a robust and specific assay that could be used to enable the purification and phenotypic properties of these cells, and to derive information about their frequency and how they are regulated. During the 3 years of this grant, I established conditions that allow human mammary gland structures to be reproducibly generated in subrenal xenografts in highly immune deficient mice, starting with small innocula of dissociated human mammary cells. The regenerated glands are similar in morphology and cellular organization to normal human mammary glands, bounded by a basement membrane with an outer layer of myo-epithelial cells and an inner layer of polarized luminal cells that can be induced to produce milk. I also established that the presence of regenerated structures can be determined by detecting the in vitro clonogenic progenitors they contain and this endpoint can serve as an objective indicator of the presence of a primitive stem-like cell in the initial cells transplanted. This retrospective functional assay allows limiting dilution analysis of positive xenograft yields to derive mammary stem cell frequencies in differently manipulated populations. Using this approach I found the frequency of stem cells in normal human mammary tissue to be ~1 per 5000 cells and their phenotype to be CD49f+ EpCAM-/low CD31- CD45-. In contrast to recent publications, I have now found that most human MRUs do not efflux Hoechst 33342 dye (implying a low/absent activity of efflux pump Brcp1/ABCG2) and also do not exhibit high fluorescence in the "Aldefluor assay" (implying a low/absent expression of aldehyde dehydrogenase). I have also developed a new method to examine the cycling activity of MRUs. These findings set the stage for further biological and molecular characterization studies of normal human mammary stem cells and their relationship to human breast cancer stem cells. Detailed findings have been published in Nature Medicine journal.

15. SUBJECT TERMS
No Subject Terms provided.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	18	USAMRMC

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INTRODUCTION

There is growing evidence to support the concept that the proliferation of many human tumors, including breast cancers, is driven by rare subpopulations of "cancer stem cells". The first event(s) that lead to the production of these cancer stem cells may occur in normal tissue stem cells, but later genetic or epigenetic changes that establish full oncogenic activity are likely to be acquired in a complex fashion over extensive periods of time in progeny with stem or progenitor features and may include the reactivation of stem cell properties in cells that had otherwise lost them. It is therefore critical to understand the mechanisms that regulate normal human mammary stem cells, and how dysregulation of these can lead to the development of breast cancer stem cells. The objective of this training grant is to develop a robust and reproducible methodology to detect, quantify and isolate stem cells in normal human mammary tissue, using a xenotransplantation system. This will enable subsequent characterization of the regulation of these important cells and comparison with breast cancer stem cells. This report covers the third year of the grant, which has focused on biological characterization of human mammary stem cells as identified by the assay methodology developed during the first two years, as well as further screening of candidate stem cell markers.

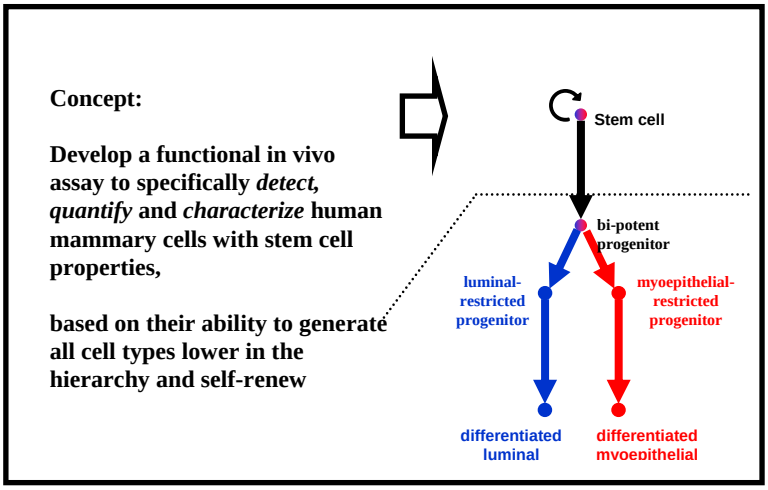
BODY

1. Concept

A number of studies in recent years have established that the mature cells in the mammary epithelium are continually generated by a multi-step differentiation process from a pool of long-lived undifferentiated self-renewing mammary epithelial stem cells[1-4]. A simplified depiction of this process is shown below, in which the two differentiated lineages (luminal, myoepithelial) are generated from stem cells via an intermediate compartment of shorter-lived, clonogenic progenitor cells. We refer to these progenitors as "colony-forming cells" (CFCs), because they are detected based on their ability to generate adherent colonies when plated at low densities in 2-D tissue cultures. We and others have recently identified and characterized more primitive and phenotypically distinct cells in the *murine* mammary gland that have *stem cell* properties, and have coined the operational term "mammary repopulating units" (MRUs) for them. This term reflects the method used to detect murine mammary stem cells which is based on their individual ability to regenerate an entire mammary tree when transplanted into the mammary stroma of a congenic recipient mouse.

The present project tests the hypothesis that an analogous stem cell population exists in the *human* mammary gland. It is very important to understand the mechanisms that regulate these cells, to investigate how dysregulation of their development/differentiation can drive the initiation and progression of human breast cancers, and to identify specific stem cell markers and molecular pathways that can potentially form the basis of future novel diagnostic and therapeutic strategies. Achievement of these ends requires the development of tools to selectively identify human mammary stem cells, as well as methodologies for purifying them from the heterogeneous populations of cells present in normal human breast tissue and eventually understanding how they are normally regulated. The objectives of this grant were to initiate this type of study.

Our strategy to identify human mammary stem cells is based on a xenotransplant system, originally developed to propagate human mammary epithelial fragments[5] and recently adapted for use with dissociated mammary cell suspensions[6]. Human mammary cells are combined with fibroblasts in small collagen gels, then implanted under the kidney capsule of highly immunodeficient, hormone-supplemented mice. After a number of weeks organized human mammary structures are regenerated in the xenografts, and these contain differentiated cells of both lineages, progenitors that can be detected in 2-D in vitro CFC assays as well as daughter cells capable of repeating this process in secondary recipients. Under the assumption that these structures, and the cells they contain, are regenerated from primitive stem-like cells ("human MRUs"), we have developed this system to act as a quantitative assay for these rare and important cells.



2. Scientific Progress

The scientific progress is summarized briefly below, with reference to the specific objectives in the Statement of Work. Greater detail is provided in the manuscript attached in the Appendix, recently published in Nature Medicine journal. References given below refer to figures in the manuscript.

Aim 1: Development and validation of a functional xenograft assay for human mammary stem cells (months 1-12)

I have established conditions that reproducibly support the generation of organized human mammary gland structures from dissociated suspensions of previously frozen normal human mammary epithelial cells placed in collagen gels that are then implanted into immunodeficient mice. The regenerated structures resemble normal human mammary tissue and contain both lineages of differentiated mammary cells with the luminal cells showing a normal polarized arrangement surrounded by myoepithelial cells bounded by a basement membrane. The luminal cells can also further mature into milk-secreting cells when the mice undergo pregnancy **(Fig. 1a, b)**.

I have also established that the number of regenerated CFCs detected in xenografts after 4 weeks in vivo serves as a sensitive and objective readout of the presence or absence of primitive MRUs present among the cells originally transplanted, allowing the frequency of MRUs in any population

to be quantified by limiting dilution approaches **(Fig. 2a-e)**. Secondary transplants have been carried out, demonstrating that MRUs self-renew as they generatecomplex glandular structures *in vivo* **(Supplementary Table 1a, b)**.

By transplanting small cell doses containing one or fewer MRUs, I have demonstrated that parent MRUs generate multiple daughter CFCs in vivo, and that the assay is therefore detecting a clonal regeneration process. I am still planning additional experiments to validate the clonality of the process, by further testing the common origin of regenerated CFCs using input human cells that can be distinguished from one another by genetic (virally-marked) and/or epigenetic (X chromosome inactivation) means.

Aim 2: Development of a robust and reproducible methodology for purifying mammary stem cells from normal primary human breast tissue (months 12-24)

I have screened a number of candidate phenotypic markers for their presence/absence on human MRUs, with the objective of identifying a combination of markers that can be used to purify stem cells from normal mammary tissue. These experiments involve measuring the MRU content in subpopulations that are FACS-sorted from reduction mammoplasty samples after staining with antibodies against various candidate stem markers. I have identified a combination of markers that are expressed by a large majority of MRUs and show a consistent pattern of expression across 9 normal mammoplasty samples. This includes a high expression of CD49f (α6 integrin), low expression of Epithelial Cell Adhesion Molecule (EpCAM), and a lack of expression of hematopoietic and endothelial markers CD45 and CD31 **(Fig. 3a-e)**. Sorting by this phenotype allows MRUs to be purified by about 10-fold compared with unsorted cells and to allow their almost complete separation from luminal restricted progenitors detected as CFCs.

I have screened other candidate phenotypic markers (CD10, Thy1, CD48, SSEA3, SSEA4, EPCR, CXCR4, CD117, CD49b, CD49d, Hoescht 33342 dye efflux), but none of these has proved useful for obtaining significant incremental enrichment beyond what has already been achieved with the $CD49f^{+}$ $EpCAM^{-/low}$ $CD31^{-}$ $CD45^{-}$ combination.

Aim 3: Biological characterization of normal human mammary stem cells and comparisons with human breast cancer stem cells (months 24-36)

I have used limiting dilution approaches to measure the frequency of MRUs in 5 different normal adult mammary tissue samples. These experiments indicate that MRUs are rare, with measured frequencies of approximately 1 per 5000 mammary cells **(Supplementary Table 1)**.

I have also carried out assays to determine whether MRUs efflux the dye Hoescht 33342 to generate a "side population" phenotype. This phenotype is a characteristic of adult murine hematopoietic stem cells but not murine mammary stem cells[3], and is believed to reflect activity of the efflux pump BCRP1/ABCG2. I found that the large majority of MRUs do *not* efflux this dye (i.e., MRUs are mostly found in the "main population").

I have also started investigating the expression in MRUs of aldehyde dehydrogenase activity as measured by the Aldefluor assay, since an $Aldefluor^{bright}$ phenotype was suggested in a recent study to characterize xeno-transplantable tumorigenic cells from human breast cancer samples[7]. My results suggest that the large majority of MRUs in normal tissue are *not* found in the $Aldefluor^{bright}$

fraction. This is interesting, as it suggests a marker that may be expressed differently between normal MRUs and the cells that generate breast tumours.

Finally, I have been developing methodologies to obtain viable subfractions from mammoplasty tissues that are enriched for cells in particular phases of the cell cycle (G0/G1/S/G2/M), using phenotypic properties that are "proxies" for cell cycle stage. These are (i) *time to enter S-phase when stimulated by growth factors in vitro*, as determined by a "suicide assay" in which cells not in G0 are killed by exposure to high specific activity ^{3}H-thymidine for the duration of a cell cycle; (ii) *cellular DNA and RNA content* as reflected in their fluorescence after staining with Hoechst 33342 and Pyronin Y; and (iii) *retention of Rhodamine*-123 after exposure of the cells to this dye. These methodologies will enable future work to identify the factors and molecular interactions that can alter the cycling status of these different normal human mammary cell types and how their effects may be dysregulated in human breast cancer stem cells.

3. Training Opportunities

I have gained hands-on experience of the various *in vitro* and *in vivo* techniques used in this project including dissociation of primary mammary tissue, flow cytometry, in vitro mammary progenitor assays and subrenal capsule surgery. I have had the opportunity to present this work orally at 4 scientific conferences, as well as give poster presentations at these and other venues. I have been involved in the preparation of a primary research paper describing the findings of this study, a protocol paper describing the methodology in detail, and have also contributed to the preparation of 3 other primary papers. I have provided instruction to 2 new doctoral students in the methods and concepts that I have learned thus far. I have also given a practical demonstration of the method at a workshop on mammary stem cell techniques.

KEY RESEARCH ACCOMPLISHMENTS

- Development and validation of a quantitative methodology to assay for human mammary stem cells
- Identification of a preliminary set of human mammary stem cell markers ($CD49f^{+}$ $EpCAM^{-/low}$ $CD31^{-}$ $CD45^{-}$) which allow a stem-cell enriched subset of cells to be isolated from adult human breast tissue
- Measurement of the stem cell frequency in normal adult human mammary tissue
- Demonstration that normal human mammary stem cells have a phenotype that is distinct from the phenotype of luminal restricted progenitors detectable in colony assays performed in vitro.
- Preliminary characterization of the activity in MRUs of efflux pumps (Brcp1/ABCG2) responsible for the Hoechst 33342 "side population phenotype" and of aldehyde dehydrogenase activity.
- Development of methodologies that can be used to sort/select subpopulations of human mammary cells that are enriched in cells in particular phases of the cell cycle (G0/G1/S/G2/M), that can form the basis of future studies to investigate the mechanisms that regulate proliferation of primitive cells in normal and malignant breast tissue.

REPORTABLE OUTCOMES

1. Peer reviewed papers

Peter Eirew, John Stingl, Afshin Raouf, Gulisa Turashvili, Samuel Aparicio, Joanne Emerman and Connie Eaves. A method for quantifying normal human mammary epithelial stem cells with *in vivo* regenerative ability. Nat Med. 2008 Dec;14(12):1384-9. Epub 2008 Nov 23.

2. Invited talks

Peter Eirew, "Identification of Human Mammary Epithelial Stem Cells With In Vivo Regenerative Ability", Canadian Stem Cell Network AGM, Vancouver, BC, November 2008

Peter Eirew, "Methods for detecting human mammary stem and progenitor cells", Talk and practical demonstration at Workshop on Mammary Stem Cell Techniques, StemCell Technologies, Vancouver, BC, June 2009

3. Abstracts

Peter Eirew, John Stingl, Afshin Raouf, Gulisa Turshvili, Sam Aparicio, Joanne Emerman and Connie Eaves, "Identification of Human Mammary Epithelial Stem Cells With In Vivo Regenerative Ability", Canadian Stem Cell Network AGM, Vancouver, BC, November 2008

REQUEST FOR NO COST EXTENSION TO AWARD EXPIRATION DATE

We have recently submitted a written request for a 12 month no cost extension to the expiration date of this award. The reason for requesting this extension is to enable the remaining funds to be used to support the principal investigator of the project (Peter Eirew) for the time he will need to complete his thesis work which include items of considerable scientific interest from the original Statement of Work that remain unfinished. These include:

- characterization of the ex vivo cytokine/growth factor response of human mammary stem cells [Aim 3c], which will provide important information about the extent to which these cells are cycling or quiescent in situ
- screening of further candidate stem cell markers with the aim of obtaining higher stem cell purities [Aim 2c],
- undertaking gene expression analysis of highly purified human stem cell populations [Aim 3e]

CONCLUSION

This project has resulted in the development and validation of a xenotransplant-based methodology to detect primitive human mammary cells (MRUs) with the hallmark features of stem cells (the ability to generate both differentiated lineages in organized 3-D structures, the ability to generate daughter CFCs, and the ability to self-renew). Notably, by combining the transplant procedure with

an endpoint "readout" after several weeks in vivo of detectable regenerated CFCs, we have established an objective, quantitative and practical way to detect MRUs in any given test population and to use limiting dilution transplants to quantify their frequency. Such experiments have shown MRUs to be rare cells in normal adult mammary tissue (1 per 5000 cells) and to be characterized by a $CD49f^{+}$ $EpCAM^{-/low}$ $CD31^{-}$ $CD45^{-}$ phenotype.

This work has been published in a very high impact journal (Nature Medicine), and presented as invited talks at various international conferences. We have also recently been invited to submit the details of our human mammary stem cell detection and quantitation methodology to another high impact journal (Nature Protocols), which will provide an excellent means to disseminate our work to the wider breast cancer research field.

We anticipate a number of benefits from this project. The establishment of a reproducible methodology to detect human mammary stem cells is considered a major breakthrough in the field, as none previously existed. When combined with markers to purify stem cells from many (though not yet all) other cell types in the breast, it allows investigations of the molecular and cellular mechanisms operating at the level of these important cells to be undertaken, avoiding problems associated with studying bulk populations. Information on how these mechanisms can become dysregulated also has the potential to lead to novel therapeutic strategies that specifically target breast cancer stem cells.

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APPENDIX

Paper published in Nature Medicine journal detailing the scientific results to date from the project funded by this Training Grant:

Eirew,P., Stingl,J., Raouf,A., Turashvili,G., Aparicio,S., Emerman,J.T., and Eaves,C.J. A method for quantifying normal human mammary epithelial stem cells with *in vivo* regenerative ability. Nat Med. 12:1384-9, 2008.

nature medicine

A method for quantifying normal human mammary epithelial stem cells with *in vivo* regenerative ability

Peter Eirew[1], John Stingl[1,6], Afshin Raouf[1], Gulisa Turashvili[2], Samuel Aparicio[2,3], Joanne T Emerman[4] & Connie J Eaves[1,5]

Previous studies have demonstrated that normal mouse mammary tissue contains a rare subset of mammary stem cells. We now describe a method for detecting an analogous subpopulation in normal human mammary tissue. Dissociated cells are suspended with fibroblasts in collagen gels, which are then implanted under the kidney capsule of hormone-treated immunodeficient mice. After 2–8 weeks, the gels contain bilayered mammary epithelial structures, including luminal and myoepithelial cells, their *in vitro* clonogenic progenitors and cells that produce similar structures in secondary transplants. The regenerated clonogenic progenitors provide an objective indicator of input mammary stem cell activity and allow the frequency and phenotype of these human mammary stem cells to be determined by limiting-dilution analysis. This new assay procedure sets the stage for investigations of mechanisms regulating normal human mammary stem cells (and possibly stem cells in other tissues) and their relationship to human cancer stem cell populations.

The human mammary gland is a compound tubulo-alveolar structure composed of two lineages of epithelial cells: an inner layer of luminal epithelial cells surrounded by an outer layer of contractile myoepithelial cells. These mature cells are in a state of constant turnover, being continually replaced from more primitive mammary epithelial progenitors. Some of these progenitors can be detected as colony-forming cells (CFCs) *in vitro*, and, in humans, luminal-restricted, myoepithelial-restricted and bipotent mammary epithelial CFCs can be prospectively isolated as separable subsets[1,2]. Definitive evidence of more primitive mammary epithelial cells with the self-renewal property of stem cells was first provided in mice by mammary fat pad transplantation experiments[3]. More recently, we and another group showed that the mammary structures produced in this assay are generated from single $CD49f^{+}CD29^{+}CD24^{low}$ mammary repopulating cells (termed mammary repopulating units, or MRUs) that are relatively rare ($\sim$1 per 1×10^3 epithelial cells) in the glands of normal adult virgin female mice[4,5].

The presence of mammary stem cells in normal adult women has been inferred from analyses of X-chromosome inactivation patterns indicating a frequent clonal origin of cells in adjacent lobules and ducts[6] and from attempts to regenerate mammary gland structures from human mammary epithelial cells (HMECs) transplanted into highly immunodeficient mice. One of these transplantation approaches has relied on colonizing the precleared mammary fat pad of such mice with human fibroblasts to create an environment conducive to the requirements of HMECs[7,8]. We have been developing an alternative strategy that involves suspending test cells together with irradiated fibroblasts in a collagen gel, which is then implanted under the kidney capsule of estrogen- and progesterone-treated nonobese diabetic severe combined immunodeficient mice (NOD-SCID) mice[9], on the basis of previous findings that viable mammary tissue fragments can be maintained at this site[10]. We now show how this latter protocol, as modified for use with dissociated human mammary cell suspensions, can be used as a quantitative assay for a subset of human mammary cells with stem cell properties and a basal phenotype.

RESULTS

Dissociated HMECs regenerate organized structures *in vivo*

We initially found that collagen gels seeded with suspensions of normal human mammary cells and irradiated mouse C3H $10T^1/_2$ fibroblasts and then placed under the kidney capsule of hormone-supplemented[11] female NOD-SCID mice (or derivative strains) contained regenerated epithelial structures when the gels were removed and examined 2–8 weeks later (**Fig. 1a**,**b**). These structures included both round and elongated duct-like arrangements of cells organized as a polarized bilayered stratified epithelium enclosing a lumen and surrounded by a basement membrane containing laminin and collagen IV (**Fig. 1b**). The cells in the inner and outer layers expressed established markers of differentiated mammary luminal and myoepithelial cells respectively. Cells expressing nuclear estrogen receptor-α and cells expressing progesterone receptors were also present. Overall, the spatial distribution of cellular markers in regenerated structures was similar to that seen in normal human mammary tissue.

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Received 3 March; accepted 6 June; published online 23 November 2008; doi:10.1038/nm.1791

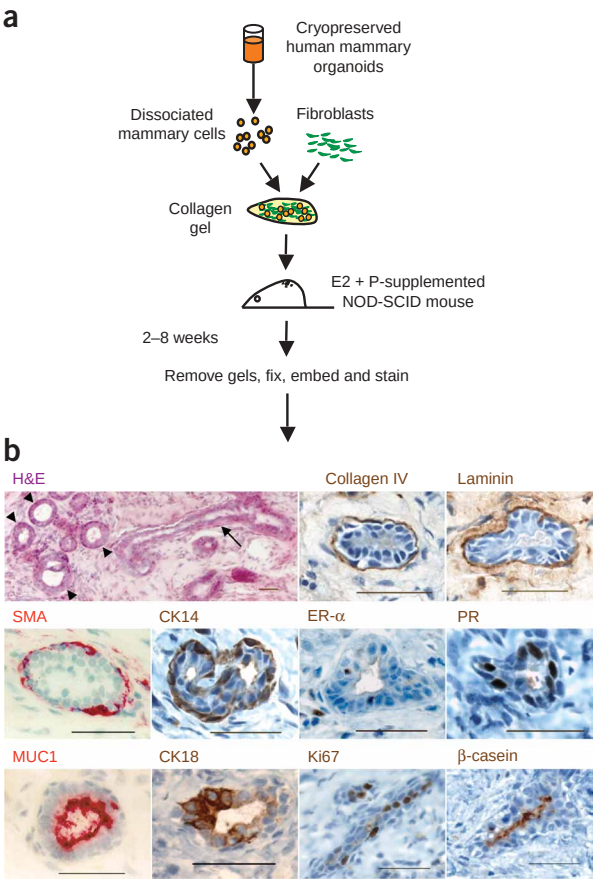

Figure 1 Organized structures are generated *in vivo* from single-cell suspensions of primary human mammary cells. (**a**) Schematic showing xenotransplantation approach. Single cells obtained by enzymatic dissociation of normal human reduction mammoplasty samples were combined with irradiated fibroblasts in a collagen gel as described in Methods. Gels were then transplanted under the kidney capsule of immunodeficient mice given slow-release pellets of human β-estradiol and progesterone (E2 + P). (**b**) H&E and immunostained sections through 4-week xenografts produced in the gels. The H&E-stained section shows examples of round (arrowheads) and elongated duct-like (arrow) structures. The immunostained xenograft sections show a spatial distribution of markers similar to that seen in normal human breast tissue. The sections stained with antibodies to collagen IV and laminin show the presence of a basement membrane separating the epithelial structures from the surrounding gel and fibroblasts. Smooth muscle actin (SMA) and cytokeratin-14 (CK14) are two markers of basally located myoepithelial cells. MUC1 and cytokeratin-18 (CK18) are luminal epithelial cell markers. The sections stained with antibodies to estrogen receptor-α (ER-α) and progesterone receptor (PR) show that some fibroblasts, as well as epithelial cells, stained positively for ER-α. Ki67 is a marker of cycling cells. The section in the bottom row at the far right is from a gel that was transplanted 4 weeks previously into a female mouse that was made pregnant 9 d after transplant. This section was stained with antibodies to β-casein, and the positive staining provides evidence of human milk production within the regenerated alveolar structure. Scale bars, 50 μm.

We also saw many cells that expressed the proliferation marker Ki67 (**Fig. 1b**) and cells with diffuse chromatin (data not shown), consistent with the interpretation that the structures are produced by an ongoing regenerative process. Some apoptotic cells were also evident (data not shown). Such structures were obtained from every human mammoplasty sample tested when at least 1×10^5 cells were transplanted.

When the female hosts were mated 1 week after the gels had been placed in the mice and the structures were examined 3 weeks later, luminal cells with vacuolated cytoplasm that stained positive for human β-casein (a protein component of human milk) were prevalent (**Fig. 1b**). They also had hyperchromic, slightly pleomorphic nuclei, typical of cells in human lactating mammary tissue (data not shown).

Regenerated CFCs serve as a read-out of transplanted MRUs

To test for the presence of mammary progenitors in the regenerated structures, we prepared single-cell suspensions from the removed gel-xenografts and plated the cells *in vitro* in two-dimensional CFC assays (**Fig. 2a**). We found all types of mammary CFCs (luminal-restricted, myoepithelial-restricted and bipotent) to be readily detectable in the xenografts for up to 12 weeks, and these CFCs grew into colonies that

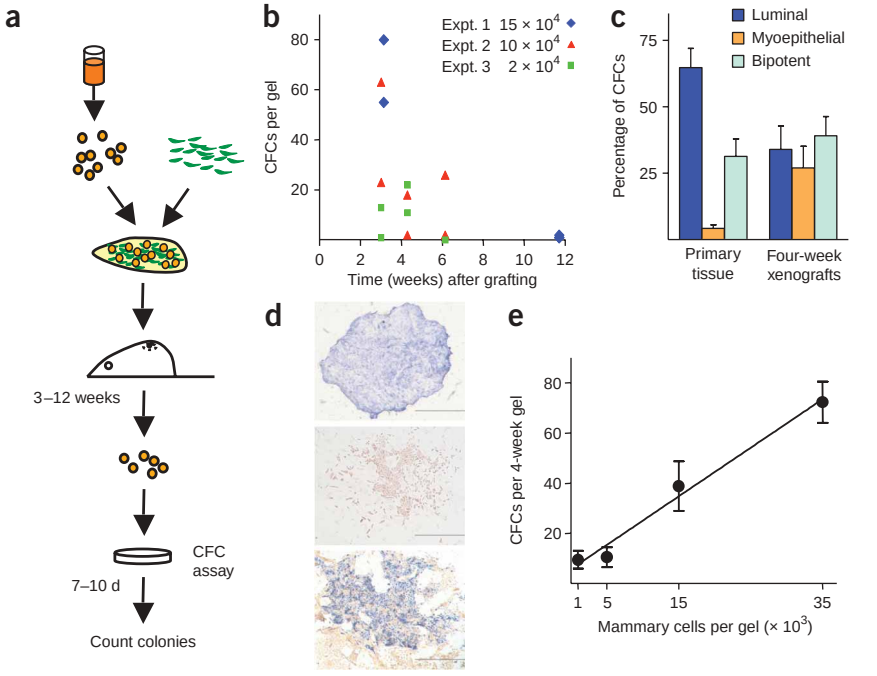

Figure 2 CFC production *in vivo* as an indicator of human MRU repopulating activity. (**a**) Experimental protocol (as in **Fig. 1a** but showing the use of CFC output measurements as an endpoint of MRU activity). (**b**) The number of CFCs detected per gel after various times *in vivo*. The legend inside the figure shows the number of human cells transplanted per gel in each of the time-course experiments performed ($n = 3$). (**c**) The distribution of different types of CFCs in freshly thawed normal human breast tissue compared with the distribution of these cells in 4-week xenografts generated from the same tissue samples ($n = 9$). (**d**) Representative colonies generated from cells derived from 4-week xenografts after dual-color immunostaining with antibodies to both MUC1 (blue) and cytokeratin-14 (brown). Top, pure luminal cell colony; middle, pure myoepithelial colony; bottom, mixed colony containing both lineages. Scale bars, 1 mm. (**e**) The CFC output in the gels after 4 weeks was linearly related to the number of human cells transplanted. Shown is a representative experiment in which six gels were analyzed per cell dose. Error bars represent means ± s.e.m.

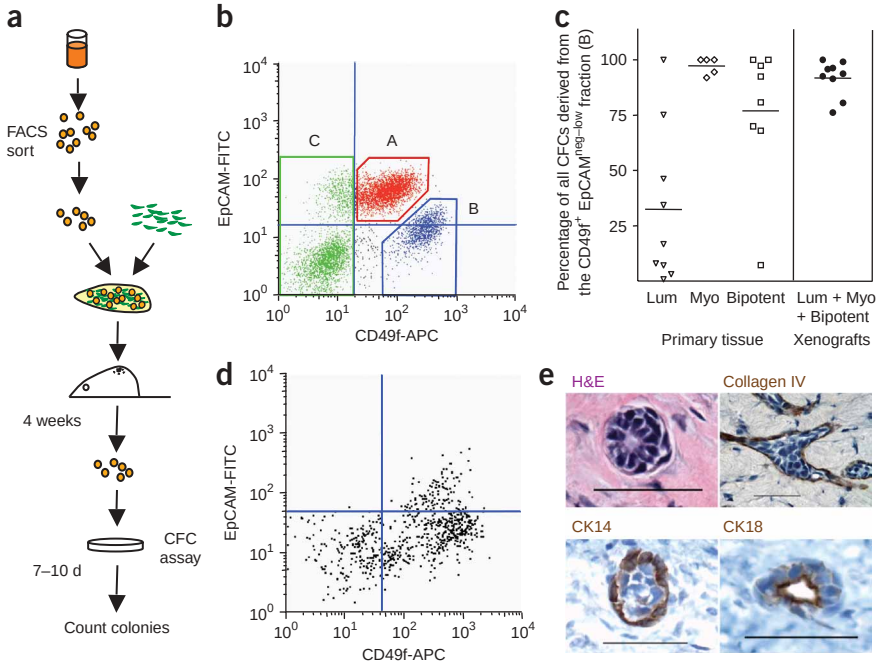

Figure 3 MRUs are CD49f$^+$EpCAM$^{neg–low}$. (**a**) Experimental protocol (as in **Fig. 2a**, but starting with FACS-isolated subsets in the transplanted gels). (**b**) FACS profile of input human mammary cell preparations (depleted of CD45$^+$ and CD31$^+$ cells) showing the gates used to select fractions assayed for MRUs and CFCs. APC, allophycocyanin. (**c**) FACS-separated fractions A, B and C as depicted in **b** were assayed *in vitro* for the presence of primary CFCs and also transplanted *in vivo* to assay for the presence of MRUs as defined by their ability to generate secondary CFCs detectable after 4 weeks. Open symbols show the proportion of each primary CFC type that was present in the original CD49f$^+$EpCAM$^{neg–low}$ fraction B ($n = 9$ mammoplasty samples tested). Lum, luminal-restricted CFCs; Myo, myoepithelial-restricted CFCs; Bipotent, bilineage CFCs. Solid circles show the proportion of subsequently detected xenograft-derived CFCs that were generated in gels originally seeded with CD49f$^+$EpCAM$^{neg–low}$ cells (fraction B). Symbols are absent where samples did not yield a given type of primary CFC. The numbers of cells assayed from each fraction were in proportion to the relative sizes of each fraction. (**d**) A representative FACS profile of cells from a 4-week xenograft initiated with CD49f$^+$EpCAM$^{neg–low}$CD31$^-$CD45$^-$ cells. (**e**) Sections of 4-week-old xenografts in gels initially seeded with purified CD49f$^+$EpCAM$^{neg–low}$CD31$^-$CD45$^-$ cells, stained with H&E (top left) or with antibodies to collagen IV (top right), CK14 (bottom left) or CK18 (bottom right). Scale bars, 50 μm.

were indistinguishable from those derived from primary mammoplasty tissue (**Fig. 2b–d**). Hereafter, we will refer to these regenerated CFCs as secondary CFCs to discriminate them from the primary CFCs present in initial suspensions of dissociated mammoplasty tissue. Transplant cell dose-response experiments further showed that the number of secondary CFCs present in xenografts after 4 weeks is linearly related to the number of human mammary cells originally suspended in the gels (**Fig. 2e**).

We then performed a series of limiting-dilution transplant experiments to determine the frequency of cells that are responsible for regenerating structures containing secondary CFCs at 4 weeks after transplant. A total of 107 gels were analyzed, each seeded with 500–60,000 cells from freshly thawed, organoid-enriched human mammary tissue (five separate experiments, **Supplementary Table 1** online). Chi-squared tests showed the results were consistent with a single-hit Poisson model[12] in each of the five experiments, supporting the interpretation that multiple secondary CFCs are derived from a single common human mammary repopulating cell or unit (human MRU). The frequency of MRUs calculated from these experiments was 1 per 1×10^3 to 1×10^4 mammoplasty cells, one to two orders of magnitude lower than the frequency of (primary) bipotent CFCs measured in the same original samples. From the frequency of MRUs determined and the total secondary CFC numbers measured, each MRU was found to generate, on average, 4.1 ± 0.6 daughter CFCs.

Human MRUs have a CD49f$^+$ EpCAM$^{neg–low}$ phenotype

We next asked whether these transplantable human MRUs belong to a phenotypically distinct subset of mammary epithelial cells. Accordingly, we isolated various subsets of cells from nine different human mammoplasty samples after staining them with antibodies to CD49f and epithelial cell adhesion molecule (EpCAM, also known as CD326; **Fig. 3a,b**). In six of the nine experiments, we simultaneously removed contaminating hematopoietic (CD45$^+$) and endothelial (CD31$^+$) cells. We plated an aliquot of each of the subsets shown in **Figure 3b** into a primary CFC assay and suspended the remaining cells in gels in numbers proportionate to their fractional yields (a total of 119 gels), and we then implanted the gels into mice. Most primary luminal-restricted CFCs (72 ± 10%) were confined to the CD49f$^+$EpCAM$^+$ fraction, whereas most primary bipotent (77% ± 11%) and myoepithelial-restricted (97 ± 2%) CFCs were concentrated in the CD49f$^+$EpCAM$^{neg–low}$ fraction (**Fig. 3c**). The CD49f$^-$EpCAM$^-$ fraction was mostly devoid of primary CFCs (data not shown). Notably, grafts in which secondary CFCs were detected 4 weeks later were almost exclusively those initiated with cells from the CD49f$^+$EpCAM$^{neg–low}$ fraction (92 ± 3% of the CFCs detected in all xenografts were obtained in gels initially seeded with CD49f$^+$EpCAM$^{neg–low}$ cells). Structures observed in these 4-week-old xenografts showed the same spectrum of CD49f$^+$ and/or EpCAM$^+$ cells detectable by flow cytometry as in primary normal human mammary tissue (**Fig. 3d**), and, upon immunohistochemical analyses *in situ*, a polarized organization of cells expressing luminal and myoepithelial markers bounded by a basement membrane (**Fig. 3e**) was again seen (**Fig. 1b**).

Because CD49f expression has been associated with basally located cells in the mouse mammary gland[13], we asked whether another marker of basal cells, CD10 (also called common lymphocyte leukemia antigen, or CALLA)[14], would be expressed on the human MRUs detected by our gel transplant assay. The results of two experiments showed that most secondary CFCs (70% in the first experiment and 86% in the second) originated from CD10$^+$ cells. However, 95 ± 3% of the CD49f$^+$EpCAM$^{neg–low}$ cells were found to be CD10$^+$, indicating that isolation of CD10$^+$ cells would not yield a purer population of MRUs.

Human MRUs can be serially transplanted

To determine whether human mammary cells defined functionally as MRUs on the basis of their *in vivo* CFC-regenerating activity also have self-renewal ability, we performed secondary transplantation assays. For these experiments, we implanted primary grafts containing

1,000–3,000 CD49f^{+}EpCAM$^{neg–low}$CD31^{-}CD45^{-} cells (MRU-enriched and containing limiting numbers of MRUs) into a first set of mice. Four weeks later, we removed the gels, prepared single-cell suspensions from them and then plated 30% of each suspension in a CFC assay to identify primary gels that contained regenerated (secondary) CFCs. We combined the remaining 70% of the cells from the primary gels with fresh feeder cells and suspended them together in new secondary gels, which we then implanted into secondary recipients (**Supplementary Fig. 1a** online). In most cases, primary gels that contained regenerated CFCs also regenerated detectable CFCs in the secondary gels, indicating that MRUs had been regenerated in the primary gels (**Supplementary Fig. 1b**). Of note, similar assays of primary gels initiated with larger numbers ($\sim 1 \times 10^5$) of cells from other (that is, MRU-depleted) fractions produced few or no CFCs in secondary recipients.

DISCUSSION

Here we describe a new, robust and objective protocol for determining the frequency of cells that meet the rigorous definition of human mammary epithelial stem cells with both *in vivo* regenerative potential and self-renewal activity demonstrable in secondary transplants. We also show that the structures that these cells produce after 4 weeks in this assay contain the same hierarchy of primitive and mature epithelial cell types as is found in the normal endogenous human mammary gland and that the regenerated cells are most frequently derived from a rare subset of cells with a distinct CD49f^{+}EpCAM$^{neg–low}$ basal phenotype. Notably, during the course of their production in this *in vivo* system, the regenerated and differentiating human mammary cells also self-organize to form a three-dimensional mammary gland structure that appears similar to normal mammary tissue and is capable of physiological maturation.

We also show that the number of CFCs in 4-week-old structures serves as a sensitive and quantitative endpoint for human mammary stem cells in the original cell suspension assayed, and their detection as an endpoint avoids the difficulties associated with reliance on a histological approach. This concept is similar to the strategy commonly used to identify very primitive subsets of mouse or human hematopoietic cells referred to as long-term culture-initiating cells by virtue of their ability to generate hematopoietic CFCs detectable after 5–6 weeks in cultures containing stromal feeder layers[15]. In the hematopoietic system, it was shown that the hematopoietic CFCs detected after 5–6 weeks must have originated from a more primitive cell type, as the cells from which they were derived had a different phenotype[16,17]. In addition, it was shown that the CFCs in the cultures were continuously proliferating and differentiating, making simple persistence an unlikely explanation for their presence[18]. Here we have also shown evidence of proliferative activity within the regenerated structures. In addition, for at least one of the mammary CFC types detected (the luminal-restricted CFCs), it was possible to show a clear difference in phenotype as compared with the cells that produced secondary mammary CFCs detectable 4 weeks later.

The ability to assay the *in vivo* mammary regenerative activity of dissociated cells is a major advance, as it enables the intrinsic developmental potential of individual cells to be investigated. It also provides renewed support for the concept that the full developmental properties of human mammary stem cells can be expressed in the absence of other cells in the epithelium, in keeping with similar findings for mouse MRUs[4,5].

EpCAM in the normal resting human breast is highly expressed by luminal epithelial cells and is less expressed by basal cells[19,20]. In contrast, CD49f (α_6 integrin) has an inverse pattern of expression[13]. Thus, the observed CD49f^{+}EpCAM$^{neg–low}$ phenotype of MRUs suggests a basal location of these cells *in situ*. Consistent with this expectation is the previous observation that most of the cells in the CD49f^{+}EpCAM$^{neg–low}$ fraction also express cytokeratin-14 (a myoepithelial marker) and not cytokeratin-19 (a luminal cell marker)[21]. In this regard, our present findings for human MRUs mirror those previously reported for mouse MRUs, which also show a basal phenotype[4,5]. In contrast, we find a marked difference between the phenotype of human MRUs and the reported CD49f^{+} EpCAM^{high} phenotype of HMECs that form branched structures in Matrigel[21], raising concerns that this Matrigel-based readout may not provide a useful surrogate assay for human mammary stem cells.

The assay described here should allow further enrichment of human MRUs to be achieved. It will also enable related studies of the biological properties and molecular regulation of MRUs of their ability to be transformed by specific oncogenes and of their relationship to cells that propagate various types of spontaneously arising human breast cancers. In this latter regard, it is noteworthy that CD49f is expressed by a subset of cells within the human MCF7 breast cancer cell line that have tumorigenic potential in immunodeficient mice[22]. We thus expect that the xenograft strategy that lies at the heart of our assay will provide a new system to investigate the mechanisms that control normal human mammary stem cell proliferation and differentiation *in vivo* and the sensitivity of these cells to agents that promote or interfere with these processes. Indeed, it may be anticipated that this *in vivo* approach will prove useful for the characterization of stem cell populations in other normal human tissues where, with the exception of the hematopoietic system, a vacuum currently exists.

METHODS

Mice. We bred and housed female NOD-SCID, NOD-SCID β2-microglobulin–null and NOD-SCID interleukin-2 receptor-γ_c–null mice at the animal facility at the British Columbia Cancer Research Centre. Unless otherwise specified, the data we present was generated with NOD-SCID interleukin-2 receptor-γ_c–null mice as transplant recipients. We carried out surgery on mice between the ages of 5 weeks and 8 weeks. All experimental procedures were approved by the University of British Columbia Animal Care Committee.

Dissociation of human mammary tissue. We collected anonymized discard tissue from normal premenopausal women (ages 19–40) undergoing reduction mammoplasty surgery with informed consent according to procedures approved by the University of British Columbia Research Ethics Board and processed the tissue as previously described[23]. Briefly, we transported the tissue from the operating room on ice, minced it with scalpels and then dissociated it for 18 h in Ham's F12 and DMEM (1:1 vol/vol, F12 to DMEM, StemCell Technologies) supplemented with 2% wt/vol BSA (Fraction V; Gibco Laboratories), 300 U ml^{-1} collagenase (Sigma) and 100 U ml^{-1} hyaluronidase (Sigma). In some experiments, this medium was supplemented with 10 ng ml^{-1} epidermal growth factor (EGF, Sigma), 10 ng ml^{-1} cholera toxin (Sigma), 1 μg ml^{-1} insulin (Sigma), 0.5 μg ml^{-1} hydrocortisone (Sigma) and 5% FBS (StemCell Technologies). We obtained an epithelial-rich pellet by centrifugation at 80*g* for 4 min and cryopreserved it in 6% dimethylsolfoxide-containing medium at –135 °C until use. We subsequently prepared single-cell suspensions from freshly thawed pellets by treatment with 2.5 mg ml^{-1} trypsin supplemented with 1 mM EDTA (StemCell Technologies), washing once with HBSS (StemCell Technologies) supplemented with 2% FBS followed by treatment with 5 mg ml^{-1} dispase (StemCell Technologies) and 100 μg ml^{-1} DNase1 (Sigma), after which we passed the cell suspension through a 40-μm filter (BD Biosciences) to remove remaining cell aggregates.

To recover cells from the xenografted gels, we killed recipient mice and aseptically removed the gels from the kidneys under a dissecting microscope. We then dissected the gels for 4.5 h at 37 °C in EpiCult-B medium (StemCell Technologies) supplemented with 5% FBS, 600 U ml^{-1} collagenase and

200 U ml^{-1} hyaluronidase. After digestion, we washed the cells once and treated them for 5 min with prewarmed tryspin-EDTA with gentle pipetting.

***In vitro* mammary colony-forming cell assay.** We incubated 60-mm tissue culture dishes for 1 h at 37 °C with a 1:43 dilution of Vitrogen 100 collagen (Collagen Biotechnologies) in PBS (StemCell Technologies). We seeded each dish with test cells obtained from primary tissue or digested collagen gels combined with 2.0 × 10^5 freshly thawed, previously irradiated (with 50 Gy) NIH 3T3 mouse fibroblast cells in 4 ml of EpiCult-B medium (StemCell Technologies) supplemented with 5% FBS and 0.5 μg ml^{-1} hydrocortisone. We incubated cultures at 37 °C and 5% CO_2, with a change to serum-free EpiCult-B plus 0.5 μg ml^{-1} hydrocortisone 1 d later. In some experiments, we replaced the EpiCult-B medium with DMEM and F12 supplemented with 0.1% BSA, 10 ng ml^{-1} EGF, 10 ng ml^{-1} cholera toxin, and 1 μg ml^{-1} insulin. After 7–10 d, we briefly fixed dishes in a 1:1 vol/vol mixture of methanol and acetone at 20 °C, stained them with Wright's Giemsa (Sigma) and visually scored the colonies under a dissecting microscope. We routinely categorized colonies into subtypes as follows: tightly-clustered cells with smooth colony boundary, luminal; dispersed teardrop-shaped cells, myoepithelial; colony containing both these elements and a ragged colony boundary, bi-lineage; although, in some cases, colonies were stained for specific human cytokeratins and human mucin-1 (MUC1) to confirm the presence of either or both of these lineages.

Preparation and assessment of collagen gels. We prepared concentrated rat's tail collagen as previously described[24] and stored it at –20 °C. We thawed aliquots and neutralized the pH immediately before use by adding two parts (vol/vol) concentrated sodium hydroxide to 78 parts concentrated collagen solution and 20 parts 5× DMEM. To prepare gels, we collected C3H 10T$^1/_2$ mouse embryonic fibroblasts (a kind gift from G. Cunha) from subconfluent cultures, X-ray irradiated them (with 15 Gy), mixed them with dissociated human mammary cells and resuspended them in cold neutralized collagen. We added 25-μl aliquots containing 2.2 × 10^5 10T$^1/_2$ cells and the desired number of human test cells into individual wells of a 24-well plate. We allowed the gels to stiffen in a 37 °C incubator for 10 min and then incubated them in warm EpiCult-B medium plus 5% FBS for 50 min. We then kept the plates on ice until all gels had been transplanted. In some of the early experiments, we used cells from a telomerase-immortalized human adult mammary fibroblast line or primary human mammary fibroblasts instead of C3H 10T$^1/_2$ fibroblasts.

Subrenal xenotransplantation surgery. We shaved the hair on the backs of anesthetized mice and swabbed the skin with 70% alcohol. We made an anterior to posterior incision approximately 1.5 cm long dorsally around the area of the kidneys. We also made a small incision in the abdominal wall above one kidney and exteriorized the kidney by applying gentle pressure on either side. Under a dissecting microscope, we lifted the kidney capsule from the parenchyma with fine forceps and made a 2–4-mm incision in the capsule. We inserted up to three gels under the capsule with a fire-polished glass pipette tip. After suturing the incision in the abdominal wall, we repeated the procedure (if required) on the contralateral kidney. Finally, we inserted a slow-release pellet containing 2 mg β-estradiol and 4 mg progesterone (both from Sigma) in MED-4011 silicone (NuSil Technology) subcutaneously in a posterior position before suturing the midline incision. This protocol was previously shown to produce sustained serum levels of these hormones in the mouse approximately equivalent to those at the human midluteal phase peak[11]. In some experiments, we mated the mice 9 d after the gels were transplanted.

Cell separation. We preblocked mammary cell suspensions in HBSS supplemented with 2% FBS and 10% human serum (Sigma), and then labeled them with an allophycocyanin-conjugated rat antibody to human CD49f (clone GOH3, R&D Systems) and FITC-conjugated mouse antibody to human EpCAM (clone VU1-D9, StemCell Technologies). In some experiments, we also labeled hematopoietic and endothelial cells with biotin-conjugated mouse antibodies to human CD45 (clone HI30, Biolegend) and human CD31 (clone WM59, eBiosciences), respectively, followed by R-phycoerythrin–conjugated streptavidin (BD Biosciences). We added propidium iodide (Sigma) at 1 μg ml^{-1} for live/dead cell discrimination. We performed all sorts on either a FACSVantageSE or a FACSDiva (Becton Dickinson).

Immunohistochemistry. We processed deparaffinized 4-μm sections of paraformaldehyde-fixed collagen gels for immunohistochemistry with a Discovery XT automated system (Ventana Medical Systems). We applied primary antibodies to estrogen receptor-α (clone 6F11, Ventana), progesterone receptor (clone 1A6, Ventana), Ki67 (clone K2, Ventana), cytokeratin-14 (clone LL002, ID Labs), cytokeratin-18 (clone Ks 18.04, Progen), laminin (polyclonal, Sigma), collagen IV (clone col94, Sigma) and β-casein (clone F14.20, Harlan Laboratories). We then applied horseradish peroxidase–conjugated Discovery Universal Secondary Antibody (Ventana) and developed the slides with the 3,3′-diaminobenzidine (DAB) Map Kit (Ventana). We processed some slides manually with primary antibodies to MUC1 (clone 214D4, StemCell Technologies) or smooth muscle actin (polyclonal, Abcam), each followed by alkaline phosphatase-conjugated Envision-AP (DAKO) and developed in FastRed (Sigma). We counterstained all slides with hematoxylin. For dual-color staining of colonies, we fixed 60-mm culture dishes briefly in 1:1 vol/vol acetone and methanol and preblocked them in Tris-buffered saline containing 5% wt/vol BSA and 10% FBS. We then incubated the dishes sequentially with an unconjugated antibody to MUC1, alkaline phosphatase–conjugated Envision-AP, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium development solution (Sigma), biotin-conjugated antibody to cytokeratin-14 (clone LL002, Labvision), horseradish peroxidase–conjugated streptavidin (Jackson Immunoresearch) and DAB.

Statistical analyses. Data are expressed as the arithmetic mean ± s.e.m. except for MRU frequencies. We calculated MRU frequencies with single-hit Poisson statistics and the method of maximum likelihood using L-Calc software (StemCell Technologies), and the values obtained are shown with the derived 95% confidence interval. We tested goodness of fit to a single-hit model using standard chi-squared statistics.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

C3H 10T$^1/_2$ mouse embryonic fibroblasts were a kind gift from G. Cunha, University of California, San Francisco. The authors acknowledge the excellent technical contributions of D. Wilkinson, G. Edin, the staff of the Flow Cytometry Facility of the Terry Fox Laboratory and the Centre for Translational and Applied Genomics. Mammoplasty tissue was obtained with the assistance of J. Sproul, P. Lennox, N. Van Laeken and R. Warren. This project was funded by grants from Genome British Columbia and Genome Canada, the Canadian Stem Cell Network and the Canadian Breast Cancer Foundation British Columbia and Yukon Division. P.E. was a recipient of a US Department of Defense Breast Cancer Research Program Studentship, a Terry Fox Foundation Research Studentship from the National Cancer Institute of Canada, a Canadian Imperial Bank of Commerce interdisciplinary award and a Canadian Stem Cell Network Studentship. J.S. held a Canadian Breast Cancer Foundation British Columbia and Yukon Division Fellowship and a Canadian National Science and Engineering Research Council Industrial Fellowship. A.R. held a Canadian Breast Cancer Foundation British Columbia and Yukon Division Fellowship and a Canadian Institutes of Health Research Fellowship. G.T. holds a Canadian Institutes of Health Research Pathology Training Fellowship. S.A. is supported by a Canada Research Chair in Molecular Oncology. The Centre for Translational and Applied Genomics laboratory is supported by a Canadian Institutes for Health Research Resource award.

AUTHOR CONTRIBUTIONS

P.E. designed and conducted most of the experiments and drafted the manuscript. J.S. initiated the work that led to the gel implant protocol, undertook preliminary experiments and contributed to the writing of the manuscript. A.R. critiqued the manuscript and participated in discussions of the experiments. G.T. and S.A. reviewed the histological preparations and contributed to the writing of the manuscript. J.T.E. helped organize the accrual of the mammoplasty material used. C.J.E. conceptualized the study and finalized the writing of the manuscript.

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A method for quantifying normal human mammary epithelial stem cells with *in vivo* regenerative ability

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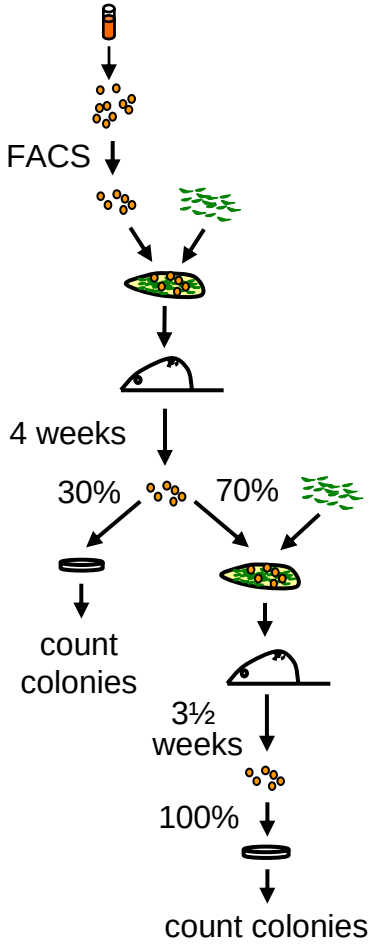
Expt no.	Recipient mouse strain	**Cells per gel**	**+ve gels/ total gels**	**MRU freq. in sample** (95% CI)	Chi squared test for consistency with 1-hit model	Average regenerated (secondary) CFCs per MRU	Primary CFC freq. in sample		
							lum	myo	bi-potent
				1 in	p=		1 in	1 in	1 in
#1	NS/IL-2Rγc$^{-/-}$	**3,000** **10,000** **25,000** **60,000**	**3/6** **5/6** **6/6** **6/6**	**4,890** (2,380-10,080)	0.99	3.7	46	706	145
#2	NS/IL-2Rγc$^{-/-}$	**1,500** **4,500** **12,000** **25,000**	**1/5** **6/6** **6/6** **6/6**	**2,220** (1,060-4,690)	0.45	5.9	38	1,500	59
#3	NS/IL-2Rγc$^{-/-}$	**500** **2,000** **7,000** **18,000**	**1/6** **5/6** **6/6** **6/6**	**1,390** (640-2,960)	0.87	2.3	151	1,059	87
#4	NS/B2m$^{-/-}$	**800** **3,000** **10,000** **22,000**	**0/6** **0/6** **3/4** **6/6**	**9,840** (4,910-19,700)	0.31	3.5	37	194	101
#5	NS	**2,000** **20,000**	**5/7** **7/7**	**1,600** (630-4,060)	1.00	5.2	43	4,000	4,000

Supplementary Table 1. Measurements of MRU frequency by limiting dilution analysis. Results from 5 experiments showing that the regeneration of CFCs in xenografted gels seeded with varying numbers of input human mammary cells 4 weeks previously fits a single hit model, indicating the origin of the CFCs from a single cell (the MRU) whose frequency can be calculated using Poisson statistics. The frequency of MRUs thus quantified was generally one to two orders of magnitude lower than the frequency of CFCs in the same initial sample. From the total number of CFCs detected in gels seeded with such derived numbers of MRUs, an average 4-week yield of 4.1 ± 0.6 secondary CFCs per input MRU was determined.

a



b

Exp't no.	Fraction *	Cells assayed	+ve / total 1ºgels (average CFC/gel)	+ve / total 2º gels (average CFC/gel)
1	B	1,280 12,800	**7/7** (15.0) **3/3** (59.0)	**6/7** (5.1) **3/3** (14.7)
	A+C	84,100	**3/3** (4.0)	**2/3** (1.0)
2	B	2,330 23,300	**7/8** (6.4) **3/3** (50.7)	**5/8** (2.2) **3/3** (61.7)
	A+C	74,800	**3/3** (2.0)	**1/3** (2.0)
3	B	1,430 14,300	**6/7** (3.0) **3/3** (30.0)	**5/6** (3.0) **2/3** (30.0)
	A+C	8,480 84,800	**1/8** (0.1) **0/3** (0.0)	**0/8** (0.0) **0/2** (0.0)

*Fraction A = $CD49f^{+}EpCAM^{+}$, B = $CD49f^{+}EpCAM^{neg–low}$, C = $CD49f^{–}$ all within the $CD45^{–}CD31^{–}$ fraction (see **Fig. 3b**)

Supplementary Figure 1. MRUs can be serially transplanted. (**a**) Experimental protocol. Cells isolated by FACS from the $CD49f^{+}EpCAM^{neg–low}CD31^{-}CD45^{-}$ (MRU-enriched) fraction or other fractions were transplanted into primary (1º) recipients. Four weeks later, 30% of the cells from each 1º gel were used to identify those that contained detectable CFCs. The remaining 70% of the cells were transplanted into secondary (2º) recipients. Another 3½ weeks later, CFC assays were performed on the cells harvested from these 2º gels. (**b**) Results. Data from 3 serial transplant experiments performed as described in (a) are shown. Both the frequency of gels implanted in 1º and 2º hosts in which at least 1 CFC was detected and, in brackets, the average number of CFCs in the assayed portion of 1º and 2º gels are indicated.